Supplementary Material

Identifying the Binding Site of Novel Methyllycaconitine (MLA) Analogs at α4β2 Nicotinic Acetylcholine Receptors

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Contents

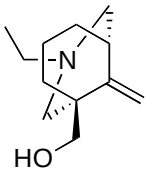
Table 1

Sense oligonucleotide primers and their respective silent mutations used to generate individual cysteine mutants in the rat α4 nAChR subunit. The underlined positions indicate the codons for cysteine while the bolded positions highlight the introduced enzyme restriction site.

α4 Mutant		Sense Oligonucleotide Primers (5'-3')	Restriction Enzyme
2'	**T278C**	CGAGAAGGTCTGCCT**ATGCAT**CTCGG	*NsiI*
6'	**S282C**	GGTCACACTGTG**CATATGC**GTGCTGCTTTCTC	*NdeI*
9'	**L285C**	CTCGGTGCT**CTGCAG**TCTCACCGTC	*PstI*
13'	**V289C**	GCTTTCTCTC**ACATGT**TTCCTGCTGC	*AflIII*
16'	**L292C**	CCGTCTTCCTGTGCC**TGATCA**CCGAG	*BcvI*

Synthetic Chemistry

General Methods. Infrared absorption spectra were obtained using a Shimadzu FTIR-84005 (Fourier Transform Infrared Spectrometer). Compounds were prepared as a thin film between 0.5 cm sodium chloride plates seated on a custom made perch in the apparatus. Absorption maxima (ν_{max}) are expressed in wavenumbers (cm^{-1}). ^{1}H Nuclear magnetic resonance spectra were recorded using a Bruker Avance 300 (300.13 MHz), Varian Gemini 300 or Varian Mercury 300 (300.06 MHz) spectrometer, and are recorded in parts per million (ppm) downfield shift from tetramethylsilane (δ_{TMS} = 0), using residual chloroform solvent (δ 7.26) or methanol (δ 3.31) as internal reference. The data is reported as chemical shift (δ_H), relative integral, multiplicity (s = singlet, br = broad, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (*J* Hz) and assignment. ^{13}C Nuclear magnetic resonance spectra were recorded using a Bruker Avance 300 (75.5 MHz), Varian Gemini 300 or Varian Mercury 300 (75.5 MHz) spectrometer at ambient temperature with complete proton decoupling. Data is expressed in parts per million (ppm) downfield relative to TMS (δ_{TMS} = 0) using deuterated chloroform (δ 77.1) as an internal reference and is reported as chemical shift (δ_C). High and low resolution mass spectra were recorded using positive electrospray ionization (ESI+) on a Finnigan PolarisQ ion trap mass spectrometer or by electron ionisation (EI) on a Micromass VG Autospec mass spectrometer. Major fragments are quoted in the form x (y), where x is the mass to charge ratio (*m/z*) and y is the percentage abundance relative to the base peak. Analytical thin layer chromatography (TLC) was performed using 0.2 mm thick aluminium backed, pre-coated silica gel plates (Merck Kieselgel 60 F_{254}). Flash chromatography was carried out using Merck Kieselgel 60 (230–400 mesh ASTM), under a positive pressure of nitrogen. Solvent compositions were mixed v/v as specified. All solvents and reagents were purified according to the methods of Perrin, Amarego and Perrin (1).



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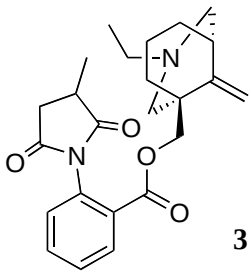

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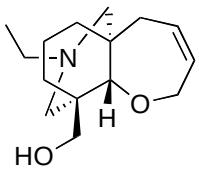

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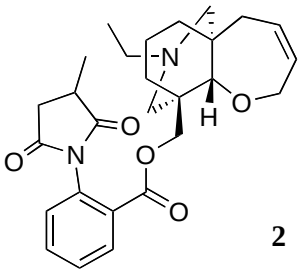
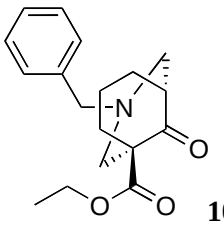


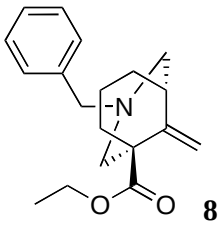
2

The synthesis of ((1*S**,5*S**)-3-ethyl-9-methylidene-3-azabicyclo[3.3.1]nonan-1-yl)methanol **5** (2, 3), (1*S**,5*S**)-3-ethyl-9-methylene-3-aza-bicyclo[3.3.1]nonan-1-yl)methyl 2-((3*R/S*)-3-methyl-2,5-dioxopyrrolidin-1-yl)benzoate **3** (2, 3), ((1*S**,7*S**,8*S**)-10-ethyl-6-oxa-10-azatricyclo[6.3.3.$0^{1,7}$]tetradec-3-en-8-yl)methanol **4** (4, 5), and ((1*R**,7*S**,8*S**)-10-ethyl-6-oxa-10-azatricyclo[6.3.3.$0^{1,7}$]tetradec-3-en-8-yl)methyl 2-((3*R/S*)-3-methyl-2,5-dioxopyrrolidin-1-yl)benzoate **2** (4, 5) have been previously reported.



10

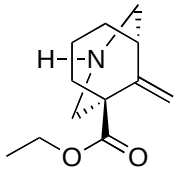
Ethyl (1R*,5R*)-3-benzyl-3-azabicyclo[3.3.1]nonan-9-one-1-carboxylate 10. To a solution of *N,N-bis*(ethoxymethyl)benzylamine (*6, 7*) (8.99 g, 40 mmol) in dry acetonitrile (50 mL) was added methyltrichlorosilane (4.7 mL, 6.02 g, 40.3 mmol). This solution was added to a solution of ethyl cyclohexanone-2-carboxylate **7** (4.57 g, 27 mmol) in acetonitrile (95 mL) and the mixture stirred at room temperature for 48 h. The reaction was quenched by the addition of sodium hydrogen carbonate solution (sat. 100 mL) and extracted with ethyl acetate (3 × 100 mL). The combined organic extract was dried over anhydrous magnesium sulfate, filtered and the solvent removed under reduced pressure to give the crude product, which was purified by column chromatography (1:19; ethyl acetate:hexane) to give the title compound **10** (6) (7.00 g, 23 mmol, 87%) as a clear colorless oil; ν_{max} 2981, 2929, 2859, 2811, 2768 (C–H), 1734, 1718 (C=O); δ_H (300 MHz, $CDCl_3$) 7.36–7.26 (5H, m, ArH), 4.19 (2H, m, OCH_2CH$_3$), 3.52 (2H, s, NCH_2Ph), 3.22–2.93 (3H, m, H2$_A$, H4$_A$, H7$_A$) 2.65–2.45 (3H, m, H2$_B$, H4$_B$, H5), 2.27–2.06 (3H, m, H6$_A$, H8), 1.66–1.58 (2H, m, H6$_B$, H7$_B$), 1.27 (3H, t, *J* 6.9, OCH$_2$CH_3); δ_C (75 MHz, $CDCl_3$) 212.7, 171.1, 138.5, 128.8, 128.6, 127.4, 62.2, 61.9, 61.3, 60.4, 59.0, 47.3, 36.8, 34.2, 20.9, 14.2; *m/z* (ESI+) 324 ($[M + Na]^+$, 10%), 302 ($[M + H]^+$, 18), 256 (100); HRMS (ESI+) found 324.1576, $C_{18}H_{23}NO_3Na$ ($[M + Na]^+$) requires 324.1576.



8

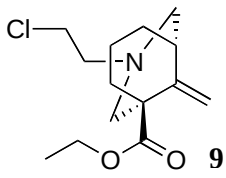
Ethyl (1S*,5S*)-3-benzyl-9-methylidene-3-azabicyclo[3.3.1]nonane-1-carboxylate 8. A solution of potassium *tert*-butoxide (5.55 g, 49.5 mmol) in dry THF (85 mL) was added to a solution of ketone **10** (3.57 g, 11.8 mmol) and methyltriphenylphosphonium bromide (8.84 g, 24.7 mmol) in THF (85 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h and allowed to warm to room temperature over the course of 24 h. The reaction was quenched by the addition of water (100 mL) and the volatile solvent removed under reduced pressure. The residual solution was washed with ethyl acetate (100 mL) and the organic layer extracted with hydrochloric acid (2 M, 3 × 80 mL). The combined aqueous extracts were cooled in an ice-bath and basified by the addition of sodium hydroxide (4 M). The organic material was then extracted into ethyl acetate (3 × 100 mL) and the combined organic extracts dried ($MgSO_4$), filtered and the solvent removed under reduced pressure

to give the crude product, which was purified by column chromatography (1:39; ethyl acetate:hexane) to give the title compound **8** (2.06 g, 6.87 mmol, 58%) as a clear colourless oil; ν_{max} 2978, 2909, 2855, 2810 (C–H), 1726 (C=O); δ_H (300 MHz, $CDCl_3$) 7.34–7.18 (5H, m, ArH), 4.76 (1H, s, C=CH_AH$_B$), 4.48 (1H, s, C=CH$_A$$H_B$), 4.26–4.15 (2H, m, NC$H_2CH_3$), 3.41 (2H, s, NC$H_2$Ph), 3.03 (1H, d, *J* 11.1, H2$_A$), 2.99–2.83 (2H, m, H4$_A$, H7$_A$), 2.63 (1H, dd, *J* 11.1, 1.8, H2$_B$), 2.41 (1H, m, H5), 2.30–2.18 (2H, m, H4$_B$, H8$_B$), 1.97–1.71 (3H, m, H6, H8$_B$), 1.57 (1H, m, H7$_B$), 1.24 (3H, t, *J* 7.2, OCH$_2$CH_3); δ_C (75 MHz, $CDCl_3$) 174.2, 151.9, 139.1, 128.6, 128.2, 126.8, 103.6, 63.1, 62.2, 60.9, 60.4, 50.6, 40.9, 35.6, 33.3, 21.6, 14.2; *m/z* (ESI+) 300 ([M + H]$^+$, 100%), 272 (22), 226 (22); HRMS (ESI+) found 300.1968, $C_{19}H_{26}NO_2$ ([M + H]$^+$) requires 300.1964.



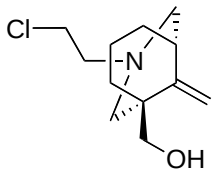
11

Ethyl (1S*,5S*)-9-methylidene-3-azabicyclo[3.3.1]nonane-1-carboxylate 11. α-Chloroethyl chloroformate (0.520 mL, 692 mg, 4.84 mmol) was added to a solution of benzylamine **8** (723 mg, 2.42 mmol) in 1,2-dichloroethane (25 mL) at 0 °C. The mixture heated at reflux for 24 h. The volatile solvent was removed under reduced pressure and the residue dissolved in methanol (25 mL), this solution was then heated at reflux for a further 24 h. The reaction was cooled, methanol removed under reduced pressure and the resultant residue dissolved in dichloromethane (20 mL) and washed with saturated sodium bicarbonate (20 mL). The aqueous layer was further extracted with dichloromethane (3 × 20 mL), and the combined organic extract dried ($MgSO_4$), filtered and the solvent removed under reduced pressure to give the crude product, which was further purified by column chromatography (acetonitrile, R_f 0.04) to give the desired compound **11** (371 mg, 1.77 mmol, 73%) as a yellow oil; ν_{max} 3431 (N–H), 2981, 2920, 2855 (C–H), 1724 (C=O); δ_H (300 MHz, $CDCl_3$) 4.51 (1H, s, C=CH_AH$_B$), 4.19 (1H, s, C=CH$_A$$H_B$), 3.90 (2H, q, *J* 6.9, OCH_2CH$_3$), 3.02 (1H, d, *J* 12.6, H2$_A$), 2.90–2.81 (2H, m, H4$_A$, H2$_B$), 2.69 (1H, dd, *J* 12.6, 3.3, H4$_B$), 2.08 (1H, m, H5), 2.04–1.95 (2H, m, H8), 1.75–1.55 (4H, m, H6, H7$_A$, NH), 1.33 (1H, m, H7$_B$), 1.00 (3H, t, *J* 6.9, OCH$_2$CH_3); δ_C (75 MHz, CD_3OD) 175.5, 152.2, 105.2, 61.7, 55.3, 53.8, 51.4, 41.3, 35.3, 33.1, 21.2, 14.6; *m/z* (ESI+) 210 ([M + H]$^+$, 27%), 164 (12), 136 (100); HRMS (ESI+) found 210.1484, $C_{12}H_{20}NO_2$ ([M + H]$^+$) requires 210.1494.



9

Ethyl (1S*,5S*)-3-(2-chloroethyl)-9-methylidene-3-aza-bicyclo[3.3.1]nonan-1-carboxylate 9. Sodium borohydride (0.265 g, 7.00 mmol) was added in three portions to a solution of chloroacetic acid (0.860 g, 9.10 mmol) in toluene (25 mL) at room temperature. After the evolution of hydrogen gas ceased, a solution of amine **11** (0.293 g, 1.40 mmol) in toluene (10 mL) was added and the reaction mixture heated at reflux for 4 h. The solution was cooled and basified with sodium hydroxide (3M, 30 mL). The organic layer was washed with a further portion of sodium hydroxide (10 mL) and the combined aqueous layers extracted with ether (3 × 30 mL). The combined organic fractions were dried ($MgSO_4$), filtered and the solvent removed under reduced pressure to give the crude product, which was purified by column chromatography (1:19, ethyl acetate:hexane) to give the desired compound **9** (0.121 g, 0.445 mmol, 32%) as a clear colorless oil; ν_{max} 2978, 2918, 2857, 2814 (C–H), 1725 (C=O); δ_H (300 MHz, $CDCl_3$) 4.75 (1H, s, C=CH_AH$_B$), 4.46 (1H, s, C=CH$_A$$H_B$), 4.16 (2H, m, OC$H_2CH_3$), 3.56 (2H, m, NCH$_2CH_2$Cl), 2.98 (2H, m, H2$_A$, H4$_A$), 2.80–2.60 (4H, m, NC$H_2CH_2$Cl, H2$_B$, H7$_A$), 2.40–2.36 (2H, m, H4$_B$, H5), 2.18 (1H, m, H6$_A$), 1.95–1.75 (3H, m, H6$_B$, H8), 1.48 (1H, m, H7$_B$), 1.26 (3H, t, *J* 7.2, OCH$_2$CH_3); δ_C (75 MHz, $CDCl_3$) 174.6, 152.3, 103.6, 62.6, 61.4, 61.3, 60.6, 56.0, 50.7, 41.1, 36.0, 33.6, 21.6, 14.4; *m/z* (ESI+) 274 ([M + H]$^+$, 10), 272 ([M + H]$^+$, 34), 236 (100); HRMS (ESI+) found 274.1395, $C_{14}H_{23}NO_2{}^{37}Cl$ ([M + H]$^+$) requires 274.1388.



6

(1S*,5S*)-(3-(2-Chloroethyl)-9-methylidene-3-aza-bicyclo[3.3.1]nonan-1-yl) methanol 6. Diisobutylaluminium hydride in toluene (0.68 mL, 1 M, 0.68 mmol) was added to a stirred solution of ester **9** (60.9 mg, 0.22 mmol) in dichloromethane (10 mL) at –78 °C, and the mixture stirred for 6 h. The reaction was quenched by the addition of sodium potassium tartrate (sat., 20 mL) and this mixture stirred overnight until the organic layer had cleared. The organic material was extracted into dichloromethane (3 × 40 mL) and the combined organic layer dried ($MgSO_4$), filtered and the solvent removed under reduced pressure to afford the crude alcohol, which was purified by column chromatography (1:4; ethyl acetate:hexane) to give the title compound **6** (40.1 mg, 0.17 mmol, 78%) as a clear colorless oil; ν_{max} 3362 (O–H), 2917, 2791 (C–H), 1648 (C=C); δ_H (300 MHz, $CDCl_3$) 4.76 (1H, s, C=CH_AH$_B$), 4.49 (1H, s, C=CH$_A$$H_B$), 3.63–3.50 (4H, m, NCH$_2CH_2Cl, CH_2$OH), 2.96 (2H, m, H2$_A$, H4$_A$), 2.80 (1H, m, H7$_A$), 2.59 (2H, t *J* 6.6, NCH_2CH$_2$Cl), 2.40–2.34 (2H, m, OH, H2$_B$), 2.18 (1H, m, H4$_B$), 1.92–1.38 (6H, m, H5, H6, H7$_B$, H8); δ_C (75 MHz, $CDCl_3$)

154.7, 101.6, 69.1, 62.5, 60.9, 59.5, 42.4, 42.0, 41.7, 36.3, 34.3, 21.5; *m/z* (ESI+) 232 ($[M + H]^+$, 31), 230 ($[M + H]^+$, 92), 163 (83), 105 (100); HRMS (ESI+) found 232.1285, $C_{12}H_{21}NO^{37}Cl$ ($[M + H]^+$) requires 232.1282; found 230.1312, $C_{12}H_{21}NO^{35}Cl$ ($[M + H]^+$) requires 230.1312.

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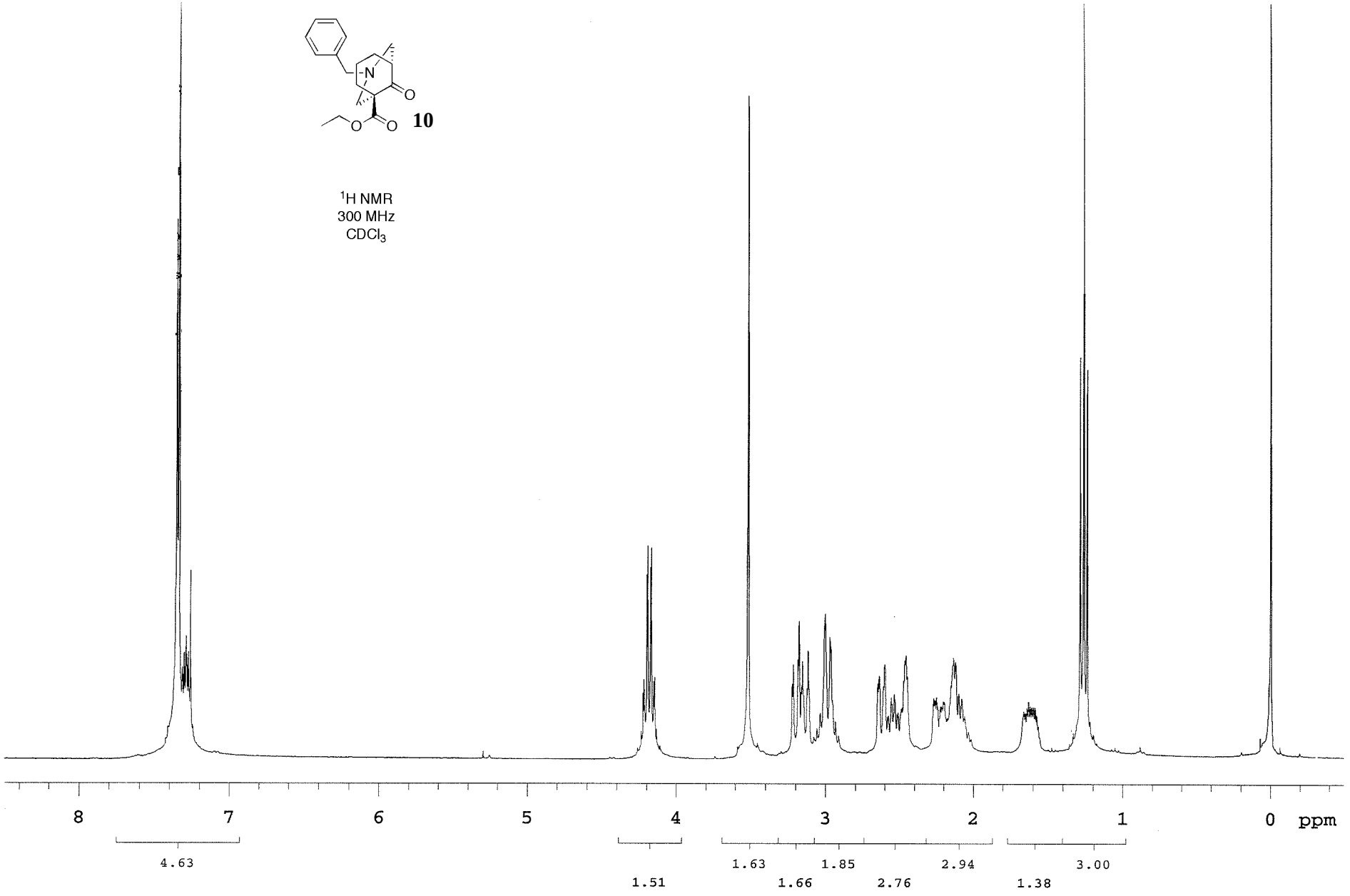
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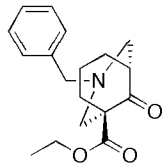
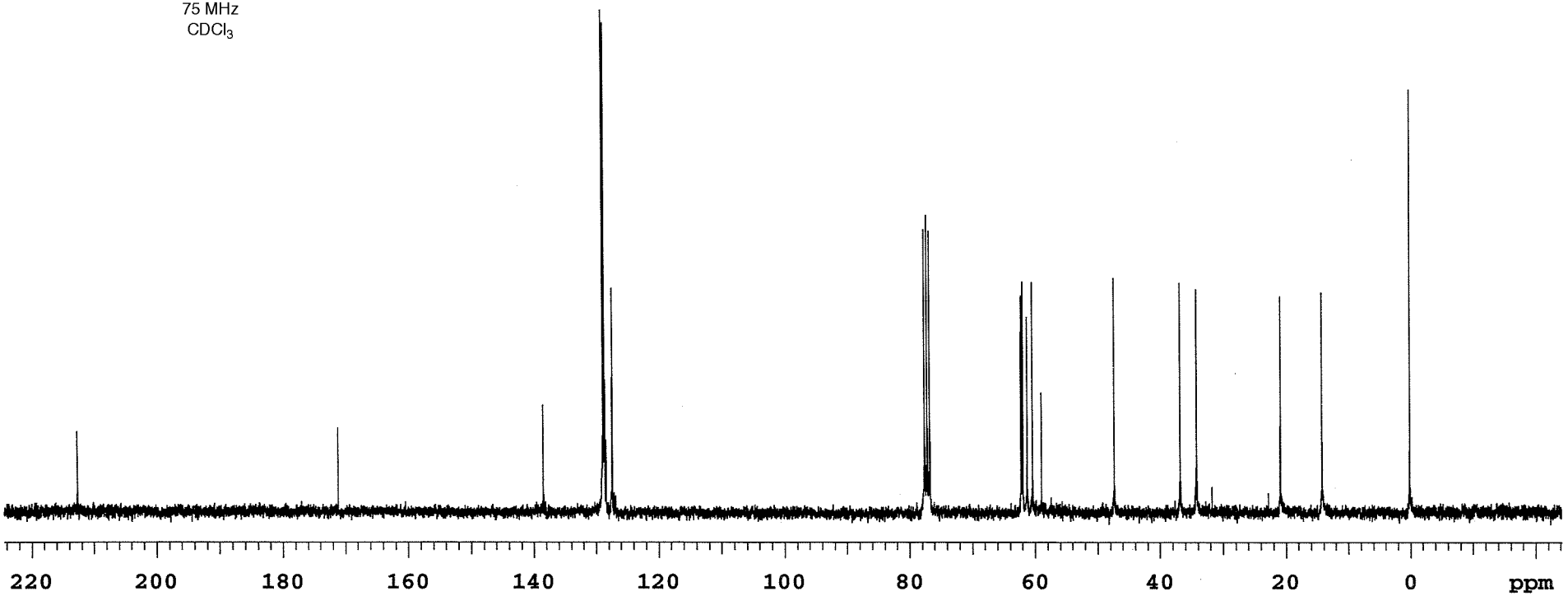
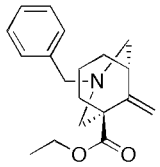
^{1}H NMR
300 MHz
$CDCl_3$
8
7
6
5
4
3
2
1
0
ppm
4.63
1.51
1.63
1.66
1.85
2.76
2.94
1.38
3.00



10

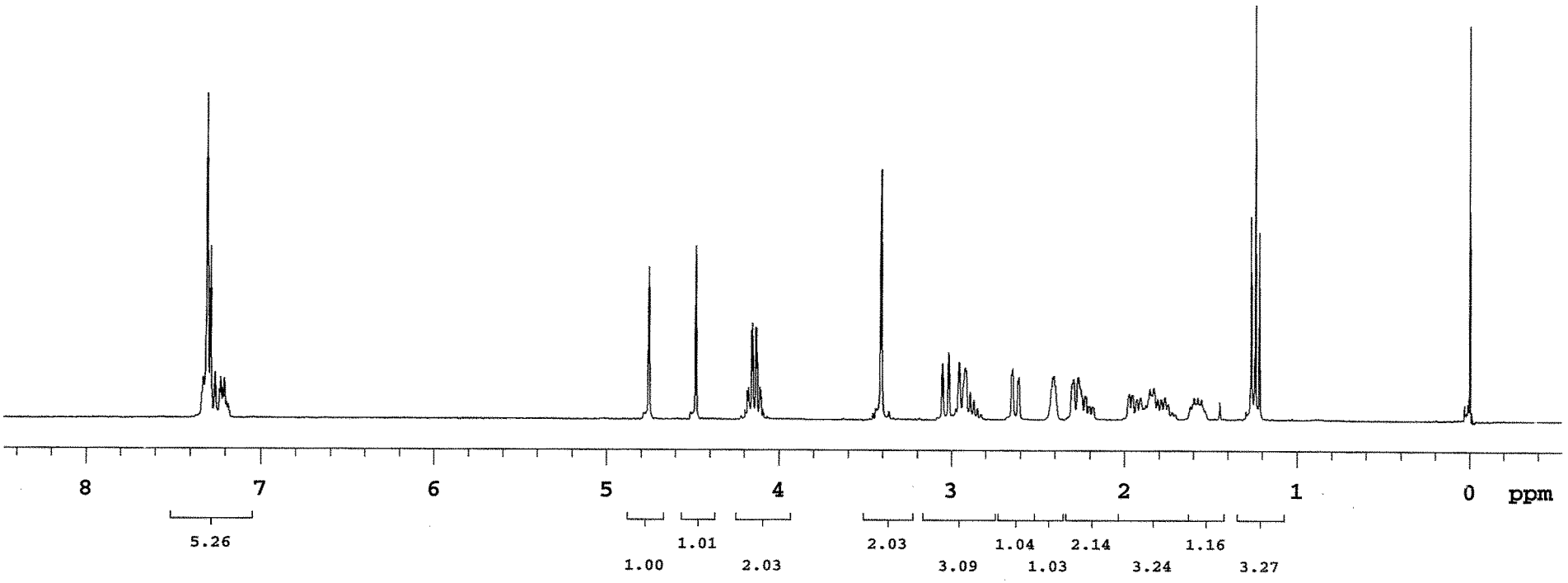
^{13}C NMR
75 MHz
$CDCl_3$

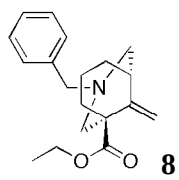




8

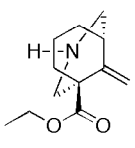
^{1}H NMR
300 MHz
$CDCl_3$





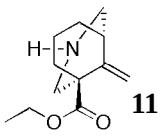
8

^{13}C NMR
75 MHz
$CDCl_3$



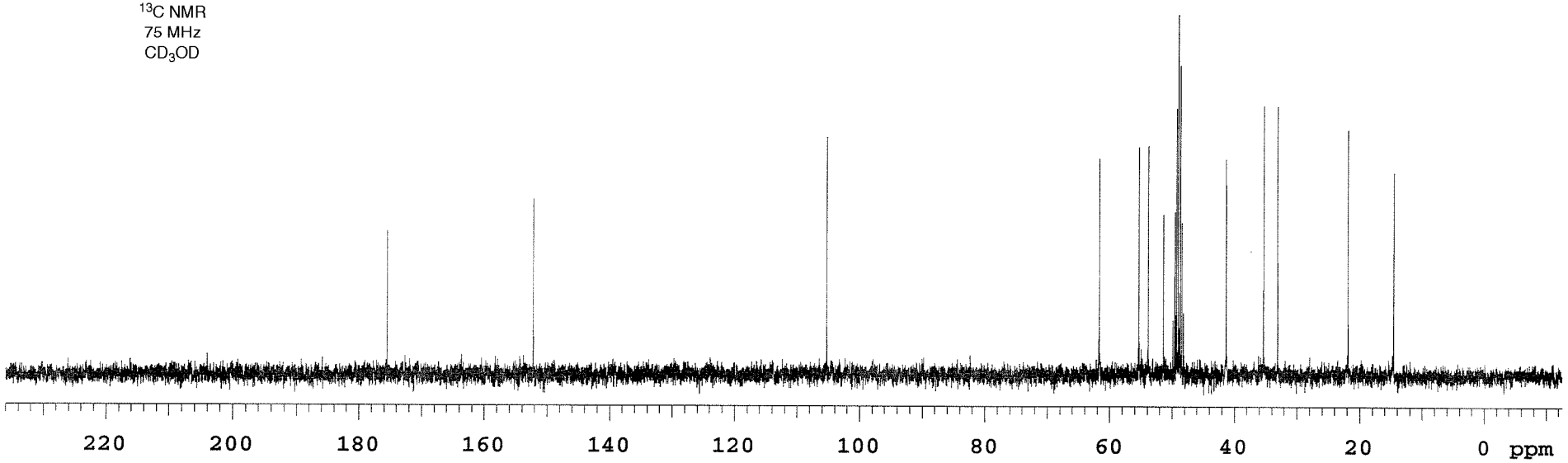
11

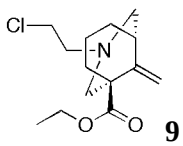
^{1}H NMR
300 MHz
$CDCl_3$



11

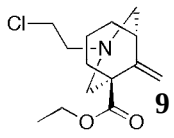
^{13}C NMR
75 MHz
CD_3OD





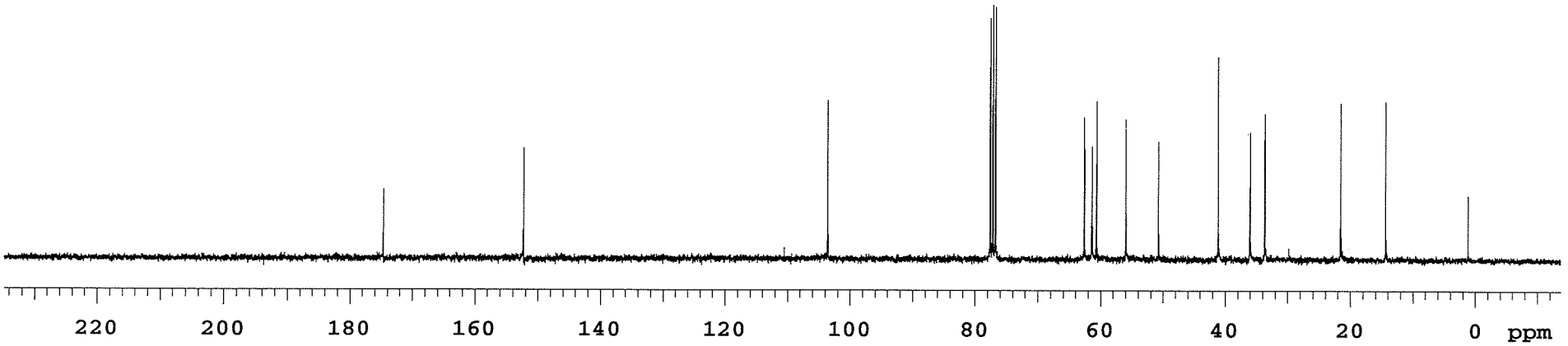
9

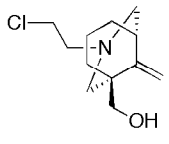
^{1}H NMR
300 MHz
$CDCl_3$



9

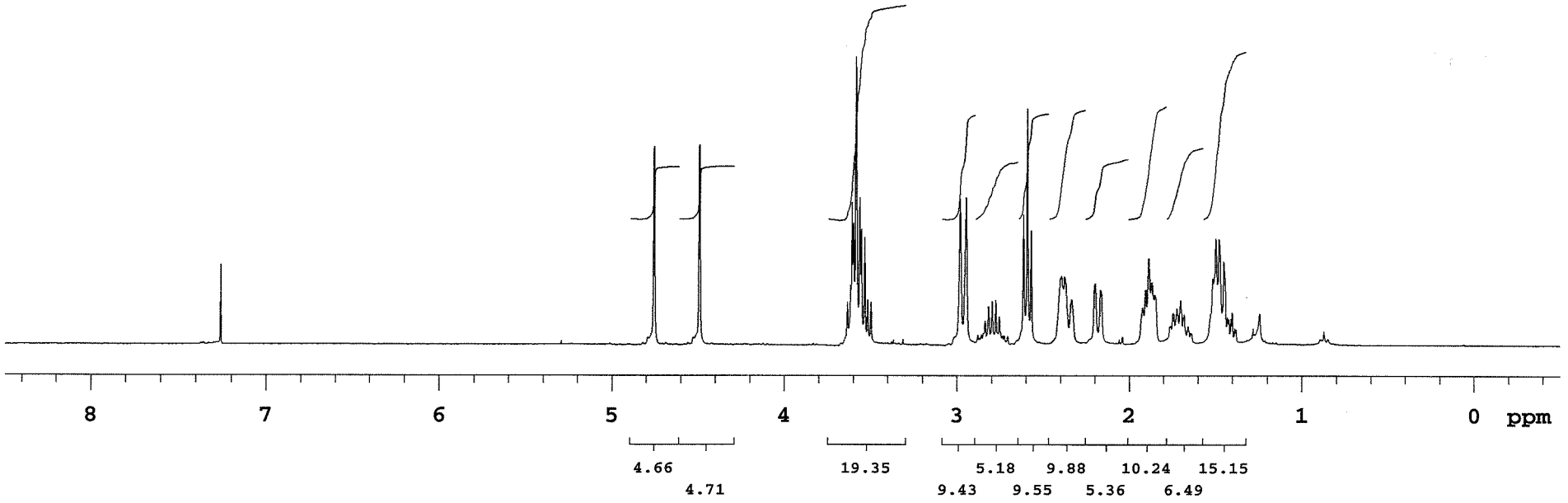
^{13}C NMR
75 MHz
$CDCl_3$

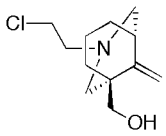




6

^{1}H NMR
300 MHz
$CDCl_3$





6

^{13}C NMR
75 MHz
$CDCl_3$

Figure S1: GOLD docking summary for flexible and rigid ligand docking of bicyclic alcohol **5.** The solutions highlighted as white text on a grey background are those used in Figures 10 and 11.

Flexible Ligand Docking

Docking Run	Operations	Fitness
1	39500	18.01
2	39500	21.00
3	39500	24.71
4	39500	16.51
5	39500	17.03
6	39500	18.68
7	39500	20.14
8	39500	19.79
9	39500	21.23
10	39500	20.03

Final ranked order of GA solutions:
3 9 2 10 8 7 6 1 5 4

RMSD Matrix of RANKED solutions

	2	3	4	5	6	7	8	9	10
1 :	0.5	2.9	1.2	1.1	1.1	6.7	5.7	6.0	2.8
2 :		3.0	0.8	1.3	0.9	6.4	5.4	5.7	2.6
3 :			3.2	3.1	3.2	6.4	5.5	5.8	3.3
4 :				1.7	1.0	6.2	5.1	5.4	2.4
5 :					1.4	6.6	5.6	5.9	3.0
6 :						6.0	5.0	5.3	2.9
7 :							1.4	1.5	7.1
8 :								0.9	6.0
9 :									6.2

```
Distance| Clusters
   0.54 |  1  2 |  3 |  4 |  5 |  6 |  7 |  8 |  9 | 10 |
   0.94 |  1  2 |  3 |  4 |  5 |  6 |  7 |  8  9 | 10 |
   0.98 |  1  2 |  3 |  4  6 |  5 |  7 |  8  9 | 10 |
   1.19 |  1  2  4  6 |  3 |  5 |  7 |  8  9 | 10 |
   1.50 |  1  2  4  6 |  3 |  5 |  7  8  9 | 10 |
   1.69 |  1  2  4  5  6 |  3 |  7  8  9 | 10 |
   3.00 |  1  2  4  5  6 10 |  3 |  7  8  9 |
   3.27 |  1  2  3  4  5  6 10 |  7  8  9 |
   7.14 |  1  2  3  4  5  6  7  8  9 10 |
```

Rigid Ligand Docking

Docking Run	Operations	Fitness
1	15500	28.88
2	15500	21.40
3	15500	25.16
4	15500	22.15
5	15500	27.75
6	15500	16.48
7	15500	22.33
8	15500	20.91
9	15500	26.41
10	15500	29.46

```
Final ranked order of GA solutions:
10  1  5  9  3  7  4  2  8  6

RMSD Matrix of RANKED solutions

        2    3    4    5    6    7    8    9   10

   1 :  0.4  0.6  9.5  2.7  5.9  6.0  2.9  3.2  2.8
   2 :       0.8  9.5  2.8  5.9  6.0  3.1  3.2  3.0
   3 :            9.3  2.7  5.6  5.7  2.9  3.1  2.7
   4 :                 9.3  4.8  4.6  9.3  8.8  9.5
   5 :                      5.7  5.8  0.5  2.4  0.6
   6 :                           0.3  5.8  5.4  5.9
   7 :                                5.9  5.5  6.0
   8 :                                     2.7  0.6
   9 :                                          2.8
```

```
Distance| Clusters
   0.32 |  1 |  2 |  3 |  4 |  5 |  6  7 |  8 |  9 | 10 |
   0.40 |  1  2 |  3 |  4 |  5 |  6  7 |  8 |  9 | 10 |
   0.54 |  1  2 |  3 |  4 |  5  8 |  6  7 |  9 | 10 |
   0.63 |  1  2 |  3 |  4 |  5  8 10 |  6  7 |  9 |
   0.79 |  1  2  3 |  4 |  5  8 10 |  6  7 |  9 |
   2.81 |  1  2  3 |  4 |  5  8  9 10 |  6  7 |
   3.21 |  1  2  3  5  8  9 10 |  4 |  6  7 |
   4.75 |  1  2  3  5  8  9 10 |  4  6  7 |
   9.54 |  1  2  3  4  5  6  7  8  9 10 |
```